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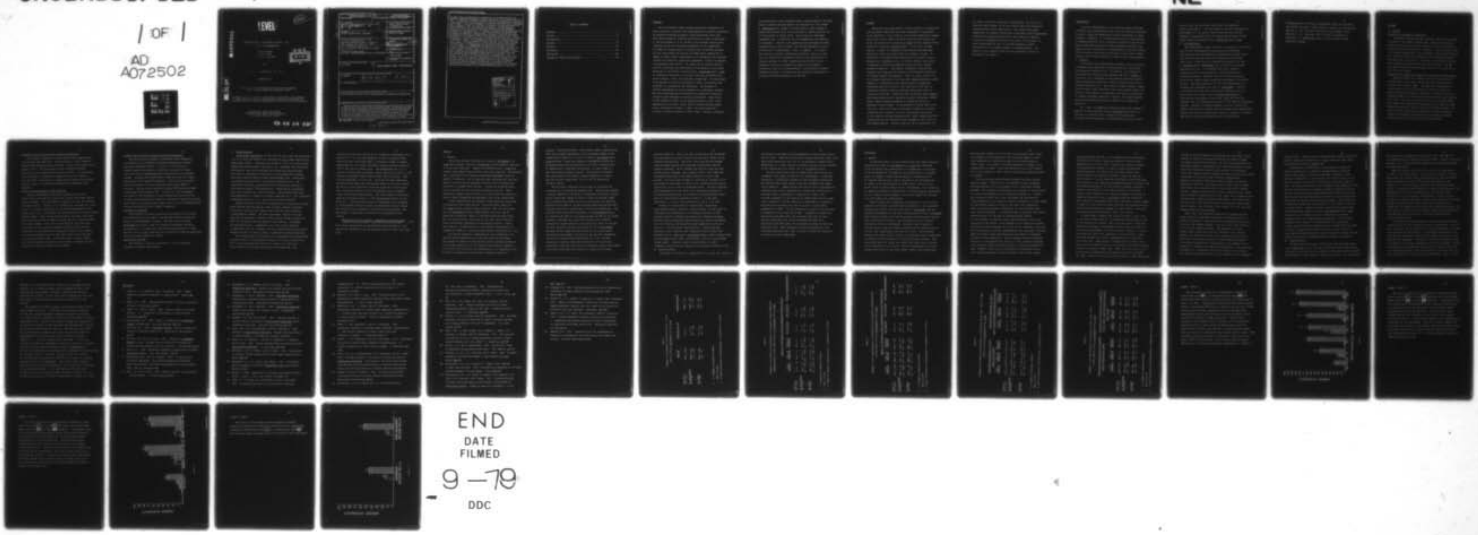

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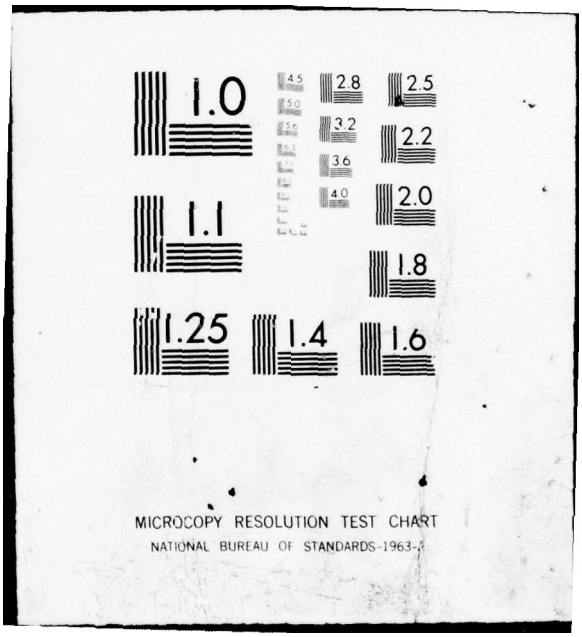
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CELLULAR IMMUNE MECHANISMS IN MALARIA AND AFRICAN TRYPANOSOMIASIS

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by

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The cell-mediated immune defense mechanisms responsible for host resistance to malaria and trypanosomiasis are poorly understood. We therefore examined peripheral blood mononuclear cells from Thai adults naturally infected with malaria in order to determine; 1) the percentages of T, B, and Null cells present during active infection and 2) the functional competence of the lymphocytes as judged by responsiveness to mitogens and cell surface antigens. We also investigated the ability of normal mouse lymphocytes to damage

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antibody coated trypanosomes as an explanation for antibody-induced cell-mediated, complement-independent, effector mechanisms of trypanosome inactivation. In comparison to healthy controls, both the percentage and concentration of peripheral T cells were decreased in individuals infected with P. falciparum and P. vivax as assessed by formation of rosettes with sheep red blood cells after 5 minutes, 1 hour, and overnight incubations. The percentage of peripheral B cells was increased but their concentration was unchanged, as assessed by two techniques: the presence of surface immunoglobulin and the presence of a complement receptor. Both the percentage and concentration of lymphocytes bearing Fc receptors were unchanged in infected individuals. Calculation of the changes in "Null" cells (defined either as non-T, non-B lymphocytes or as non-T, non-B, non-Fc receptor bearing lymphocytes) revealed an increase in "Null" cell percentage but a decrease in absolute number of "Null" cells. Finally, peripheral blood mononuclear cells exhibited normal responsiveness to mitogens and cell surface antigens despite the decrease in T cell number. T. rhodesiense were capable of being lysed by either immune or normal mouse spleen cells in the presence of immune serum but not in the presence of normal serum. The killing was complement independent and, in addition, was not augmented by the addition of complement. Finally, both lymphocyte and macrophage enriched populations were able to effect the antibody dependent cellular cytotoxicity of trypanosomes. Thus, in summary: 1) in adult Thai patients naturally infected with malaria, there is a real loss of circulating T lymphocytes with no real change in B, Fc receptor bearing, or "Null" lymphocytes and with no loss of lymphocyte function as judged by mitogenic and antigenic stimulation; and 2) in trypanosomiasis, cell-mediated antibody-dependent killing of the parasites may provide an additional or alternative defense mechanism for the host.

TABLE OF CONTENTS

Abstract

The cell-mediated immune defense mechanisms responsible for host resistance to malaria and trypanosomiasis are poorly understood. We therefore examined peripheral blood mononuclear cells from Thai adults naturally infected with malaria in order to determine: 1) the percentages of T, B, and Null cells present during active infection and 2) the functional competence of the lymphocytes as judged by responsiveness to mitogens and cell surface antigens. We also investigated the ability of normal mouse lymphocytes to damage antibody coated trypanosomes as an explanation for antibody-induced cell-mediated, complement-independent, effector mechanisms of trypanosome inactivation. In comparison to healthy controls, both the percentage and concentration of peripheral T cells were decreased in individuals infected with P. falciparum and P. vivax as assessed by formation of rosettes with sheep red blood cells after 5 minute, 1 hour, and overnight incubations. The percentage of peripheral B cells was increased but their concentration was unchanged, as assessed by two techniques: the presence of surface immunoglobulin and the presence of a complement receptor. Both the percentage and concentration of lymphocytes bearing Fc receptors were unchanged in infected individuals. Calculation of the changes in "Null" cells (defined either as non-T, non-B lymphocytes or as non-T, non-B, non-Fc receptor bearing lymphocytes) revealed an increase in "Null" cell percentage but a decrease in absolute number of "Null" cells. Finally, peripheral

blood mononuclear cells exhibited normal responsiveness to mitogens and cell surface antigens despite the decrease in T cell number. T. rhodesiense were capable of being lysed by either immune or normal mouse spleen cells in the presence of immune serum but not in the presence of normal serum. The killing was complement independent and, in addition, was not augmented by the addition of complement. Finally, both lymphocyte and macrophage enriched populations were able to effect the antibody dependent cellular cytotoxicity of trypanosomes. Thus, in summary: 1) in adult Thai patients naturally infected with malaria, there is a real loss of circulating T lymphocytes with no real change in B, Fc receptor bearing, or "Null" lymphocytes and with no loss of lymphocyte function as judged by mitogenic and antigenic stimulation; and 2) in trypanosomiasis, cell-mediated antibody-dependent killing of the parasites may provide an additional or alternative defense mechanism for the host.

Preface

During the past eight months, we have worked in collaboration with two Walter Reed Army Institute of Research investigators: 1) Dr. Bob Wells, in the study of the cellular immunologic changes which occur in Thai adults naturally infected with malaria and 2) Dr. Ray Perry in the study of in vitro cellular immune effector functions in African Trypanosomiasis. In the study of human malaria, a number of fundamental observations have been made which will orient our future work for a number of years. In particular, individuals with malaria have been found to have a decrease in T lymphocytes yet, in initial studies, have normal T lymphocyte function as judged by mitogen and cell surface antigen stimulation. Furthermore, preliminary observations indicate that these patients exhibit serum mediated suppression of mitogen induced blastogenesis. Thus, in future studies, it will become important to learn how serum immunoregulatory factors capable of influencing T cell function and/or T lymphocyte subclasses might be related to the inability of the host to completely eliminate malaria parasites during the course of recurrent infections. During the past two months, major changes have taken place with Dr. Bob Wells recently returning to WRAIR and Dr. Mike Gilbreath preparing to replace Dr. Wells in Thailand in three months. Dr. Gilbreath will spend time in our lab in St. Louis on his way to Thailand in order to learn new techniques and to prevent a loss of experimental continuity. In the study of African Trypanosomiasis, major changes have also taken place with Dr. Ray Perry being assigned to the office of the Surgeon General. Because there will be no replacement for

Dr. Perry, and we will therefore, unfortunately, not be able to continue these studies, all of our efforts have been directed at completing the initial observations. The finding that mouse lymphocytes will mediate destruction of antibody coated Trypanosomes, coupled with in vivo observations of the transfer of immunity in mice by B lymphocytes points to the need, in the future, to better understand the role of antibody dependent cellular cytotoxicity in the immune response to Trypanosomiasis. The results of our studies to date will be described in detail in the body of the report.

Introduction

A better understanding of cell mediated immune mechanisms in malaria and trypanosomiasis may allow more rational approaches to ways of improving immunological defense mechanisms in these diseases. Therefore, it is hypothesized that the investigation of subpopulations of cells in patients clinically infected with malaria and characterization of the mechanisms involved in *in vitro* cellular cytotoxicity of trypanosomes will provide insight into the control and effector functions of the cellular immune system's role in host resistance to malaria and trypanosomiasis.

A. Malaria:

It has been well demonstrated in animals, that circulating antibodies can be passively transferred and will provide protection against malaria (1-3). In addition, the transfer of resistance with cells has been demonstrated for *P. berghei* in rats (4,5) with a subsequent study demonstrating that the most likely mechanism by which cell transfer worked was related to antibody production (6). Thus, based on animal studies, lymphocutes are most likely involved in protection against malaria by virtue of their ability to synthesize and secrete antibody and to regulate antibody production. However, the immune response to an active malaria infection in humans is not completely understood.

The changes in lymphocyte populations have been studied in African children infected with *P. falciparum* (7,8) but it is unclear as to whether these findings can be generalized for other malaria parasites to infected adults living in other

areas or what the functional significance of lymphocyte alterations might be. For this reason we chose to determine the percentages of T, B, and Fc receptor bearing cells and the response of unseparated cells to mitogens and antigens in Thai adults naturally infected with P. falciparum and P. vivax.

B. Trypanosomiasis

The immunological events involved in resistance to infection with African trypanosomes are only partially understood. Resistance has been experimentally transferred by immune serum (19) and by B-enriched spleen cell populations from immune donors (10). Since the cells transferring immunity for both T. gambiense and T. rhodesiense, are in the B cell enriched population (11-14), it is probable that cells capable of synthesizing and secreting specific antibody provide the protection observed. However, the final effector mechanism by which the immune system eliminates trypanosomes is currently unclear. The work of Diggs et al (15) has demonstrated that antibody plus complement will kill T. rhodesiense. Recently, it has also been shown that normal human lymphoid cells are able to kill the culture form of Trypanosoma dionisii (a stercorarian trypanosome of bats) when coated with specific antibody (16), as indicated by release of ^{51}Cr labelled cytoplasmic protein. Similarly, antibody dependent cellular cytotoxicity (ADCC) effected by rat spleen cells has recently been demonstrated against the culture (epimastigote) form of T. cruzi (17). However, ADCC has not been demonstrated against the blood forms

(trypamastigotes) of African trypanosomes, which are the forms that exhibit antigenic variant specific surface coat glycoprotein and are, in fact, the only forms known to be present in the human host. We, therefore, felt it would be important to determine if T. rhodesiense could be lysed through an ADCC mechanism in vitro.

Methods

A. Malaria

Isolation of mononuclear leukocytes:

Peripheral blood mononuclear leukocytes (MNL) were obtained according to the method of Boyum (18). Heparinized blood was drawn from each malaria patient immediately before treatment or 14 days after the initiation of treatment. The blood was diluted 1:2 in Hanks Balanced Salt Solution (HBSS, Grand Island Biological Co. (GIBCO) New York) and layered on ficoll-Hypaque (Pharmacia Fine Chemicals, New Jersey). Following centrifugation, the MNL were removed by Pasteur pipette from the interface. The MNL were then adjusted by hemocytometer count to a concentration of 2×10^6 cells in HBSS.

Preparation of sheep red blood cells for rosette forming assays:

Sheep red blood cells (SRBC) in Alsever's solution were filtered with sterile gauze and washed with thriethanolamine-buffered salt solution (TBS). The SRBC were resuspended at a concentration of $7\text{-}8 \times 10^9$ cells/ml in TBS containing 0.1% gelatin (Baltimore Biological Laboratory, Baltimore, Maryland). Unmodified SRBC (E rosttes) were used to determine the percentage of T cells. In order to detect cells with Fc receptors, SRBC were coated with subagglutinating amounts of 7S (IgG) anti-SRBC (Cordis Laboratories, Miami, Florida) to form EA rosettes. In order to detect cells bearing complement receptors, SRBC were coated with subagglutinating amounts of 19S (IgM) anti-SRBC (Cordis) and then incubated with fresh mouse serum.

Procedures for detecting rosette forming lymphocytes:

The methods of Mendes et al were employed with modifications as previously described (19). The percentage of cells forming E rosettes was determined after 5 minutes, 1 hour, and 18 hours (overnight) at 4^{o} C. The percentage of cells forming EA and EAC rosettes was determined after 30 minutes at 37^{o} C. In calculating the percentages of lymphocytes forming rosettes with three or more red cells, both sides of a hemocytometer chamber were counted and the values of rosetting and nonrosetting lymphocytes were averaged.

Direct fluoresceinated antibody technique:

Surface immunoglobulin bearing B lymphocytes were also identified by staining with fluorescein labelled anti-human-immunoglobulin (GIBCO). The methodology of Chess et al (19) was employed with modification. Briefly, 3-4 x 10^{6} MNL were incubated for 1 hour at 37^{o} C to remove non-specifically adsorbed immunoglobulin (20), washed three times, centrifuged, and fluoresceinated anti-human immunoglobulin added. After mixing and incubation, the cells were again centrifuged and washed in HBSS containing 10% heat inactivated fetal calf serum (GIBCO). After final centrifugation, the cells were resuspended in cold glycerol phosphate buffered saline and the percentage of fluorescing cells determined using a Leitz ortholux microscope equipped with BG 12 and K 530 filters with a 220 watt high pressure mercury lamp. As with the rosette assays, counts were done blind with the sample origin unknown, readings were done in duplicate, and the results were averaged.

Mitogen and cell-surface antigen induced blastogenesis:

The proliferative response of peripheral blood lymphocytes to mitogens was investigated by standard techniques as we have previously described (21-23). In brief, 1 x 10^5 cells for mitogen studies were placed in triplicate in microtiter plates with either media, phytohemagluttinin, concanavalin A or pokeweed mitogen. Cultures were pulsed after 4 days with 0.05 ml of media containing 0.2 uCi methyl-^{3}H-thymidine. After 4 hours in culture with the thymidine, the cells were harvested using a MASH II extractor. One-way mixed lymphocyte cultures (MLC) were performed as previously described (21, 23). In brief, 2 x 10^5 responding cells and 2 x 10^5 mitomycin-C treated stimulating cells in 0.2 ml of final culture medium were placed in triplicate in microtiter plates. After 6 days, the cultures were pulsed with 0.2 uCi of ^{3}H-thymidine for 16 hours and harvested with a MASH II apparatus.

Treatment of patients:

The treatment of malaria patients participating in this study was administered by the staff of the National Malaria Eradication Project at Pradubbhabaht, Thailand. Patients infected with P. falciparum were given 1 gram of sulfadoxine (Fansidar) and 50 mg pyrimethamine in a single dose and 15 mg primaquine/day for 4 days. Patients with P. vivax infections were treated with 1500 mg chloroquine and 15 mg primaquine/day for 5 days.

Statistical analyses:

The Student's t test was used and $p < .01$ was considered necessary to obtain "significance".

B. Trypanosomiasis:

Trypanosome rhodesiense, the Wellcome CT strain (24) was maintained in 6 to 10 week old C57BL/6 mice obtained from the Jackson Laboratories (Bar Harbor, Maine). Organisms were stored at -70° C in tissue culture medium 199 (Microbiological Associates, Inc., Bethesda, MD) containing 10% glycerol; the frozen organisms then thawed and passed through mice prior to experiments; and finally the organisms were isolated from mouse blood by DEAE cellulose chromatography (25). Enumeration of organisms was carried out as previously described (15). For immunization purposes, trypanosomes were irradiated by exposure to 60 K rads utilizing a ^{60}CO gamma source. Organisms are immunogenic after this treatment but cannot multiply. Inbred C57BL/6 mice were hyperimmunized by a regimen of 5 weekly injections of 1×10^7 irradiated trypanosomes and were bled 7 days after the final injection.

Immune and normal spleen cell suspensions were prepared by mincing mouse spleens and pressing the tissue through a 60-mesh stainless steel grid; the cells were washed three times and then counted in a hemocytometer chamber. For some experiments, spleen cells were separated by adherence to plastic tissue culture flasks incubated at 37° C for 30 minutes and the adherent cells used as macrophage enriched populations. Nonadherent cells were further depleted of phagocytic cells by treatment with iron carbonyl and density centrifugation (21,22) in order to obtain a lymphocyte enriched population.

The incorporation of ^3H-leucine into trypanosomes was accomplished by modifications of the methods of Diggs et al (15) by incubation at 37° C for 2 hours with ^3H-leucine (10 μ ci/ml) in a mixture of equal volumes of 5% glucose and leucine free medium 199. The

cytotoxicity assay was carried out by incubating trypanosomes for 2 hours at 37° C in a 5% CO_2 atmosphere in 400 µl microcentrifuge tubes with heat inactivated (56° C, 30 minutes) normal or immune mouse serum with or without complement (guinea pig serum stored at -70° C) and with or without cells. Two hundred and ten µl of a mixture of serum and/or complement, and/or spleen cells with 1×10^6 labelled trypanosome targets made up each reaction mixture. Each test was performed in triplicate. After the two hour incubation, microcentrifuge tubes were centrifuged (1000 x g) for four minutes and 50 µl of each supernatant fluid was placed on a filter paper disc, dried and counted in 10 ml of Econofluor using a Packard liquid scintillation spectrometer. The total amount of ^3H-leucine added was counted by drying 1×10^6 trypanosomes directly on the paper disc. Distilled water lysis of trypanosomes was considered to induce maximal leucine release and spontaneous leucine release was estimated after incubation in normal mouse serum alone. The percentage of trypanosome cytotoxicity was determined by the formula:

$$\frac{\text{Experimental leucine release - spontaneous leucine release}}{\text{Maximal leucine release - spontaneous leucine release}} \times 100$$

Statistical significance was determined by using Student's t test and the Null hypothesis was rejected when values of p were less than 0.05.

Results

A. Malaria

Forty-nine patients infected with either P. falciparum or P. vivax were studied. The 24 P. falciparum patients ranged in age from 17 to 48 years (mean 25). Twenty-five patients with P. vivax were studied, who ranged in age from 16 to 45 years (mean 24). The patients were inhabitants of the region surrounding Prabuddabaht which is endemic for malaria. All of these patients indicated that they had had one or more previous episodes of malaria but no records were available to validate their history. A group of 21 healthy Thais with no previous history of malaria served as controls. These individuals ranged in age from 19 to 45 years (mean 32) and were of similar ethnic background and geographic area as the study group.

Patient parasitemias and complete blood counts were monitored in conjunction with these studies. The parasitemia values for the vivax patients ranged between "positive" and 1.5% while those for the falciparum patients ranged between "positive" and 3%. Hematologic evaluation (Table 1) revealed slight decreases which were not statistically significant in the total white blood cell counts and percent lymphocytes in the malaria patients. There was, however, a marked decrease in the absolute concentration of lymphocytes (Table 1), which was statistically significant ($p < .01$).

Circulating lymphocyte subpopulations were studied by E, EA, and EAC rosette formation and fluorescein labelled anti-human-immunoglobulin (FITC) staining and the results expressed either as a percentage (Table 2) or as an absolute concentration (Table 3). To determine the percentage and concentration of T lymphocytes, the E rosette technique was employed with incubation times of 5

minutes, 1 hour and 18 hours. The 5 minute values indicated that there was a marked suppression in the percentage (Table 2) and concentration (Table 3) of T cells in both P. falciparum and P. vivax patients. There was likewise a reproducible pattern of a decreased percent and concentration of E rosette forming cells (T lymphocytes) in patients with malaria, at assay times of 1 hour and 18 hours (Tables 2 and 3). The decrease in percent and concentration of E rosette forming lymphocytes (T cells) was statistically significant in 10 of the 12 comparisons (Tables 2 and 3).

The EA rosette technique was utilized in evaluating the percentage of circulating Fc bearing cells. Patient cells showed virtually identical mean values in comparison with normal controls and no significant change in terms of either percentage (Table 2) or concentration (Table 3) of Fc bearing cells was observed. Application was made of the EAC rosetting technique and the FITC staining technique in identifying circulating B lymphocytes. The mean EAC values were 25% for patients with falciparum malaria as compared with 24% for the vivax patients and 16% for the normal controls. The FITC cell values for patients were likewise elevated and showed excellent correlation with those for the EAC (B cell) rosettes with mean percentages of 24 for falciparum patients, 22 for vivax patients and 15 for the normal group. The elevated percentages of B cells in the patients compared to controls were statistically significant ($p < 0.01$) for both techniques. However, when the concentration of B cells was calculated, there was essentially no difference found in the number of B cells in patients with malaria in comparison to normal

controls (Table 3). Null cells were calculated as the percentage and concentration of non-T, non-B cells and non-T, non-B, non-Fc receptor bearing cells. The B cell value used was the average value obtained from the two techniques (fluorescence and EAC rosette formation), the Fc receptor bearing cell value was from the EA rosette technique, and a separate "Null" cell number was calculated using each of the three different E rosette time periods. As can be seen in Table 4, there was an increase in the percentage of both types of "Null" cells, which was statistically significant for 6 of the 12 calculated values. In direct contrast, upon determination of the "Null" cell concentration (Table 5). we found decreased values in the malaria infected patients which were not statistically significant using $p < .01$ but were significant in 5 out of 12 calculations at the $p < .05$ level.

Because of the decrease in circulating T lymphocytes noted during malaria infection, described above, we then began to investigate the abnormalities in lymphocyte function in Thai patients naturally infected with malaria by examining *in vitro* mitogen induced lymphocyte responsiveness as a general assessment of T cell function and allogeneic mixed lymphocyte culture as a specific T cell functional characteristic. Preliminary data obtained to date indicates that lymphocytes from 15 adult Thai patients with *P. vivax* and *P. falciparum* exhibit normal responsiveness to phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). Likewise, normal responsiveness in mixed lymphocyte culture has been observed in 10 patients examined so far.

B. Trypanosomiasis

In Figure 1 the effect of varying killer to target cell ratios on

the killing of antibody coated Trypanosomes by normal mouse spleen cells is seen. There was no killing of trypanosomes when normal cells plus normal serum were used, but in the presence of immune serum a significant ($p < 0.05$) increase in percent cytotoxicity was observed at all (except the 10:1) spleen cell to trypanosome ratios.

We next examined the roles of immune mouse spleen cells and complement in in vitro cytotoxicity of trypanosomes. As was seen previously, a significant increase in the percentage cytotoxicity was observed when normal spleen cells were incubated with immune serum (Figure 2). Immune spleen cells did not kill trypanosomes by themselves and did not significantly increase the cytotoxicity of antibody coated trypanosomes in comparison to normal cells. Although complement in the presence of immune serum led to a significant increase in killing, addition of complement to incubation mixtures which included cells did not result in significant augmentation of ADCC. We next determined if spleen cells depleted of phagocytic cells could effect ADCC. Lymphocyte enriched cell populations were 95% viable and contained less than 1% latex particle ingesting cells, while macrophage enriched cell populations were 95% viable and contained an average of 58% latex ingesting cells. Both lymphocyte enriched and phagocytic cell enriched populations were able to effect cytotoxicity (Figure 3) of antibody coated trypanosomes.

Discussion

A. Malaria

As described above, we have observed that Thai adults naturally infected with either P. falciparum or P. vivax have a decrease in the percentage and concentration of T lymphocytes, an increase in the percentage but no change in the concentration of B lymphocytes, no change in either the percentage or concentration of Fc receptor bearing cells, and an increase in the percentage but no change in the concentration of either non=T, non-B or non-T, non-B, non-Fc receptor bearing cells. Furthermore, T cell function, as judged by lymphocyte responsiveness to three mitogenic lectins and to cell surface antigens appears to be intact despite the decrease in T cell number and concentration.

These findings are in agreement and disagreement with various aspects of previously reported results by Wyler (7), who studied 30 children and 3 adults in West Africa with P. falciparum, and Ade-Serrano and Osunkoya (8), who also studied children with P. falciparum. Both previous studies (7,8) and the present study found that during acute infection with malaria, the percentage of cells forming E rosettes (T cells) was decreased. Both the study of Wyler (7) and the present study also found a decrease in the concentration of T cells, thus signifying a true loss of T cells. Both previous studies (7,8) found similar results to the present study in observing an increase in the percentage of B cells using EAC rosettes. More importantly Wyler (7) found, as we did, that there was no increase in the concentration of B cells. There is disagreement as to the changes which occur in the non-T, non-B ("Null") cell populations.

Both previous studies (7, 8) (as well as our present study) found an increase in the percentage of the calculated number of "Null" cells and Wyler (7) found an increase in the concentration of "Null" cells. In contrast, we found essentially no change in the concentration of "Null" cells and therefore do not find evidence for a true increase in "Null" cells in the patient population which we studied.

The similarities and differences between the three studies deserve comment. First of all, the decrease in both percentage and concentration of T cells appears to be a consistent finding. Secondly, the increase in percentage with no change in concentration of B cells also appears to be a consistent observation. Finally, the increase in both "Null" cell percentage and concentration found previously (7) is different from our findings of no real change in the absolute number of cells in the "Null" cell population. There are several possible explanations for the different "Null" cell results including: 1) techniques used to determine the surface characteristics, 2) the ages of the populations studied, 3) the incidence of previous infections with malaria, and 4) the locations of the studies. With regards to the techniques employed, we determined the percentage of B cells by two techniques, detecting both the presence of surface immunoglobulin and complement receptors, while the other studies determined only the number of complement receptor bearing lymphocytes. Either approach should be adequate because it is recognized that there is a close correlation between the complement receptor and the presence of surface immunoglobulin (26). However, there may be a difference in techniques in that our control percentage of B cells is 15% $\pm$ 2.3% (surface immuno-

globulin bearing) and 16% ± 1.0% (complement receptor bearing), while Wyler (7) found a normal percentage of B cells in his population to be 31.5% ± 2.3%. Another very likely difference is the population studied, in that our study was done with adults (mean age: 25 years) while the other studies were done primarily with children (in Wyler's study the mean age was 2.5 years). A third important difference is that there was some evidence in our study (by undocumented patient histories) that the subjects had had one or more previous episodes of malaria. Finally, the location of the study could play a role in the results either because of race (Asian as opposed to African) or subtle differences in the P. falciparum organisms themselves. Thus, technical differences resulting in different baseline numbers in addition to the study of adults from Asia who had had previous infections with malaria as opposed to young children from Africa, could explain the differences in "Null" cell results.

We next need to examine the question of what a "Null" cell is in terms of these studies. As originally defined (and as used in the previous two studies) "Null" cells are non-T, non-B cells and their percentage is determined by subtracting the number of T cells plus the number of B cells from 100. However, it is now clear that the "Null" cell population is heterogeneous. These cells may develop into or be a subpopulation of B cells (22) or T cells (27). Functionally, a subpopulation of "Null" cells which bear Fc receptors function as effectors of antibody dependent cellular cytotoxicity (ADCC) (28). These same cells may function as effectors of spontaneous cell mediated cytotoxicity (SCMC) (17, 29). Because the crucial surface receptor in ADCC and perhaps

in SCMC is the Fc receptor, we determined the percentage of cells bearing Fc receptors by doing EA rosettes and found no change in subjects infected with malaria. We feel that determining the potential changes of lymphocytes with a defined receptor of known functional significance may be more important for evaluating "Null" cell changes than the subtraction method, which could reflect alterations in any one of many different subpopulations. Moreover, by using an additional surface receptor, we are now potentially left with a new population of "Null" cells (perhaps "Null-Null" cells) which are non-T, non-B, and non-Fc receptor bearing. Whether the definition of non-T, non-B or non-T, non-B, non-Fc receptor bearing cells was used, there was no change in cell concentration in malaria infected individuals. More importantly, the only "Null" cell related receptor of functional significance, the Fc receptor, was not found to be a marker of a cell type that changed during malaria infection.

While the T cell depression observed in infected patients could represent a transient relocation of lymphocyte pools, it would be reasonable to hypothesize other explanations related to an active host immune response. Loss of T cells could be due to destruction by lymphocytotoxins. The depression in T cell numbers might reflect the loss of a specific T cell subclass. Finally, the ability of T cells or Fc receptor bearing cells to participate in cellular cytotoxic reactions might be enhanced or depressed during active infection due to the changes in cell populations. Whatever the underlying mechanism(s), the depression in numbers of T lymphocytes may not be limited to circulating cell populations but may involve T cell depletion of the lymphoid

organs as well. Indeed, Krettli and Nussenzweig reported depletions in mouse lymphocyte populations in thymus glands and lymph nodes during infection with P. berghei (30).

Following the classification of the changes occurring in relative proportions of lymphocyte subpopulations as observed above we have begun to study the functional competence of the patient's lymphocytes. In particular, the loss of T cells in the patients raises the question of which T cell functions might be lost. The observations above that peripheral blood lymphocytes from patients with malaria will undergo mitogenesis induced by lectins and cell surface antigens (mixed lymphocyte culture) demonstrates that T cells from infected patients are able to be non-specifically stimulated and specifically sensitized in vitro. The possibility is therefore raised that the decrease in T cell number is related to serum factors. Indeed preliminary studies have demonstrated that pooled serum samples from 16 P. falciparum and 15 P. vivax patients have an inhibitory effect on PHA and Con A induced mitogenesis but not on PWM induced mitogenesis. Since cellular competence with regards to mitogenesis does not necessarily reflect cellular effector competence future studies will be needed in order to examine the ability of patient T cells to kill in cell mediated lymphocytotoxicity and of patient Fc receptor bearing cells to function in antibody dependent cellular cytotoxicity.

B. Trypanosomiasis

We have used the release of ^{3}H-leucine from the blood stages of T. rhodesiense to test for ADCC mediated by murine spleen cells. Cytotoxicity was observed with immune but not normal serum and could be effected by either normal or immune spleen cells. No cytotoxicity

was observed with immune cells and normal serum. The addition of complement did not augment the observed ADCC. Finally, cytotoxicity appeared to be effected by both lymphocyte enriched and macrophage enriched populations.

ADCC has recently been shown to be active in vitro against a number of infectious agents. In an extensive series of studies, Butterworth and coworkers have demonstrated that human peripheral blood eosinophils will kill ^{51}CR labeled Schistomula in the presence of sera from patients infected with Schistosoma mansoni (31-33). Diamond has described ADCC of Cryptococcus neoformans (34) and Mkwanazi et al (16) and Sanderson et al (17) have shown ADCC activity in vitro against Trypanosomes. Finally, Torten et al (35) have reported the ADCC of target cells coated with bacterial membrane components and we have found that peripheral blood lymphocytes are capable of killing antibody coated meningococci (36). On the basis of these and other studies, Waksmann has hypothesized that ADCC may play a major role in the host defense against infectious agents (37).

In the studies described above, we have also examined several characteristics of cell mediated killing of trypanosomes. First of all, we did not find any situations in which mononuclear cells alone were cytotoxic for the bacteria. In particular mononuclear cells from multiple immunized animals did not mediate destruction of Trypanosomes by themselves. Secondly, we found that complement would not enhance the killing by mononuclear cells plus antibody. Finally, both adherent and nonadherent cells served as efficient effectors of trypanosome killing so that the possibility that more than one type of mechanism is involved must be entertained. That is, since

antibody is co-cultured with the target in the *in vitro* reaction mixtures, it is clearly possible that macrophages may have first opsinized the trypanosomes, followed by phagocytosis and then intracellular killing. On the other hand, non-phagocytic cells are more likely to have mediated cytotoxicity through cell to cell contact.

The importance of ADCC *in vivo* in host defense against African trypanosomes is unclear. It has been clearly shown by Diggs et al (15) that antibody dependent complement mediated cytotoxicity is a highly efficient mechanism of killing of blood forms of African trypanosomes (15). However, the studies of Diggs and coworkers are of particular interest in that they have demonstrated that a variety of effector mechanisms may be involved. Not only have they shown that antibody plus complement will cause cytotoxicity against *T. rhodesiense* (15), but also recent data from their laboratory (Diggs, C. L.: personal communication) has shown: 1) that the alternative pathway of complement activation is sufficient to support cytotoxic activity against antibody coated *T. rhodesiense* and 2) that in C5 deficient or C3 depleted mice trypanosomes are neutralized by antibody normally. Therefore, *in vivo* neither the classical nor the alternative pathways are required to be intact for antibody induced killing of *T. rhodesiense*. These observations suggest a mechanism of resistance to trypanosome infection which is independent of the complement system. It is therefore possible that both complement and cell mediated antibody dependent killing occur, thus providing alternative mechanisms of immunological defense to the host.

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Table 1

Changes in Peripheral Blood White Cells in Thai Adults with *P. falciparum* and *P. vivax*

	WBC/mm^3	Lymphocytes (%)	Lymphocytes/mm^3
Controls* (N = 21)	7567 ± 472†	38 ± 2.1	2800 ± 214
P. falciparum (N = 24)	6485 ± 345	31 ± 2.0	1942 ± 158
	(p < .1)‡	(p < .05)	(p < .01)
P. vivax (N = 25)	6831 ± 320	29 ± 2.1	1948 ± 160
	(p < .2)	(p < .05)	(p < .01)

* N = Number of subjects studied.

† Mean ± S.E.M.

‡ p value for malaria infected group in comparison to control.

Table 2

Changes in the Relative Percentages of Peripheral Blood Lymphocyte Subpopulations in Thai Adults with *P. falciparum* and *P. vivax*

	Percent of Peripheral Blood Lymphocytes Identified by					
	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes	EA rosettes	EAC rosettes	Surface Immunoglobulins
Controls (N = 21)*	41 ± .9†	54 ± .9	63 ± .3	5 ± .3	15 ± .2	15 ± .5
P. falciparum (N = 24)	28 ± 1.0	41 ± 1.0	52 ± .5	5 ± .5	25 ± .5	24 ± .6
	(p < .001)‡	(p < .001)	(p < .001)	(p < .9)	(p < .001)	(p < .001)
P. vivax (N = 25)	29 ± 1.0	41 ± 1.0	54 ± .7	5 ± .3	24 ± .5	22 ± .5
	(p < .001)	(p < .001)	(p < .001)	(p < .9)	(p < .001)	(p < .001)

* N = Number of subjects studied,

† Mean ± S.E.M.

‡ p value for malaria infected group in comparison to control.

Table 3

Changes in the Concentration of Peripheral Blood Lymphocyte Subpopulations in Thai Adults with P. falciparum and P. vivax

	Number of Peripheral Blood Lymphocytes per mm^3 Identified by					
	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes	EA rosettes	EAC rosettes	Surface Immunoglobulin
Controls (N = 21)*	1162 ± 475†	1486 ± 154	1750 ± 136	137 ± 17	447 ± 35	400 ± 26
P. falciparum (N = 24)	564 ± 56	781 ± 69	999 ± 85	100 ± 13	486 ± 38	467 ± 34
	(p < .02)‡	(p < .001)	(p < .001)	(p < .1)	(p < .5)	(p < .2)
P. vivax (N = 25)	557 ± 46	1086 ± 311	1058 ± 96	97 ± 10	483 ± 37	429 ± 33
	(p < .01)	(p < .3)	(p < .001)	(p < .05)	(p < .5)	(p < .5)

* N = Number of subjects studied.

† Mean ± S.E.M.

‡ p value for malaria infected group in comparison to control.

Table 4

Changes in the Relative Percentages of Peripheral Blood "Null" Cells in Thai Adults with *P. falciparum* and *P. vivax*

	Calculated percentage of non-T, non-B cells using:			Calculated percentage of non-T, non-B, non-Fc receptor bearing cells using:		
	5'E rosettes	1 hr. E rosettes	18 hr. E rosettes	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes
Controls (N = 21)*	43.4 ± 0.9†	30.2 ± 0.8	22.1 ± 0.5	38.7 ± .9	25.5 ± 1.0	17.4 ± .5
P. falciparum (N = 24)	46.6 ± 1.0	35.1 ± 1.0	23.8 ± 0.5	41.6 ± .9	29.8 ± 1.0	18.8 ± .5
	(p < .05)‡	(p < .001)	(p < .05)	(p < .05)	(p < .01)	(p < .1)
P. vivax (N = 25)	47.9 ± 1.2	36.5 ± 1.2	23.6 ± 0.6	43.3 ±1.2	31.4 ± 1.2	18.6 ± .6
	(p < .01)	(p < .001)	(p < .1)	(p < .01)	(p < .001)	(p < .2)

* N = Number of subjects studied.

† Mean ± S.E.M.

‡ p value for malaria infected group in comparison to control.

Table 5

Changes in the Concentration of Peripheral Blood "Null" Cells in Thai Adults with *P. falciparum* and *P. vivax*

	Calculated concentration (cells/mm^3) non-T, non-B cells using:			Calculated concentration (cells/mm^3) of non-T, non-B, non-Fc receptor bearing cells using:		
	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes
Controls (N = 21)*	1205 ± 94†	832 ± 61	627 ± 59	1070 ± 80	695 ± 50	490 ± 45
P. falciparum (N = 24)	942 ± 85	713 ± 67	489 ± 44	841 ± 76	617 ± 58	385 ± 33
	(p < .05)‡	(p < .2)	(p < .1)	(p < .05)	(p < .3)	(p < .1)
P. vivax (N = 25)	945 ± 86	720 ± 69	467 ± 40	850 ± 40	621 ± 78	365 ± 32
	(p < .05)	(p < .3)	(p < .05)	(p < .1)	(p < .4)	(p < .05)

* N = Number of subjects studied.

† Mean ± S.E.M.

‡ p value for malaria infected group in comparison to control.

Legend - Figure 1

The percentage cytotoxicity of trypanosomes incubated in normal mouse serum  or in hyperimmune mouse serum  with normal mouse spleen cells in varying ratios to the trypanosomes of from 1:1 to 100:1. Trypanosomes lysed by distilled water released 55.2% of incorporated ^{3}H-leucine as compared with the total leucine release found by drying trypanosomes on to filter paper disc. Trypanosomes lysed by distilled water were not infective to mice and thus were equated to 100% cytotoxicity. Vertical bars represent $\pm$ the standard error of the mean for five experiments. Cytotoxicity due to immune serum and normal spleen cells was significantly different from that due to normal serum and normal cells in all cell to trypanosome ratios except the 10:1 ratio. Each individual cytotoxicity assay contained 100 µl heat inactivated serum (Δ 56^{o} C for 30 minutes), 100 µl spleen cells (in medium 199) and 10 µl of ^{3}H-leucine labelled trypanosomes (1 x 10^{6}/10 µl).

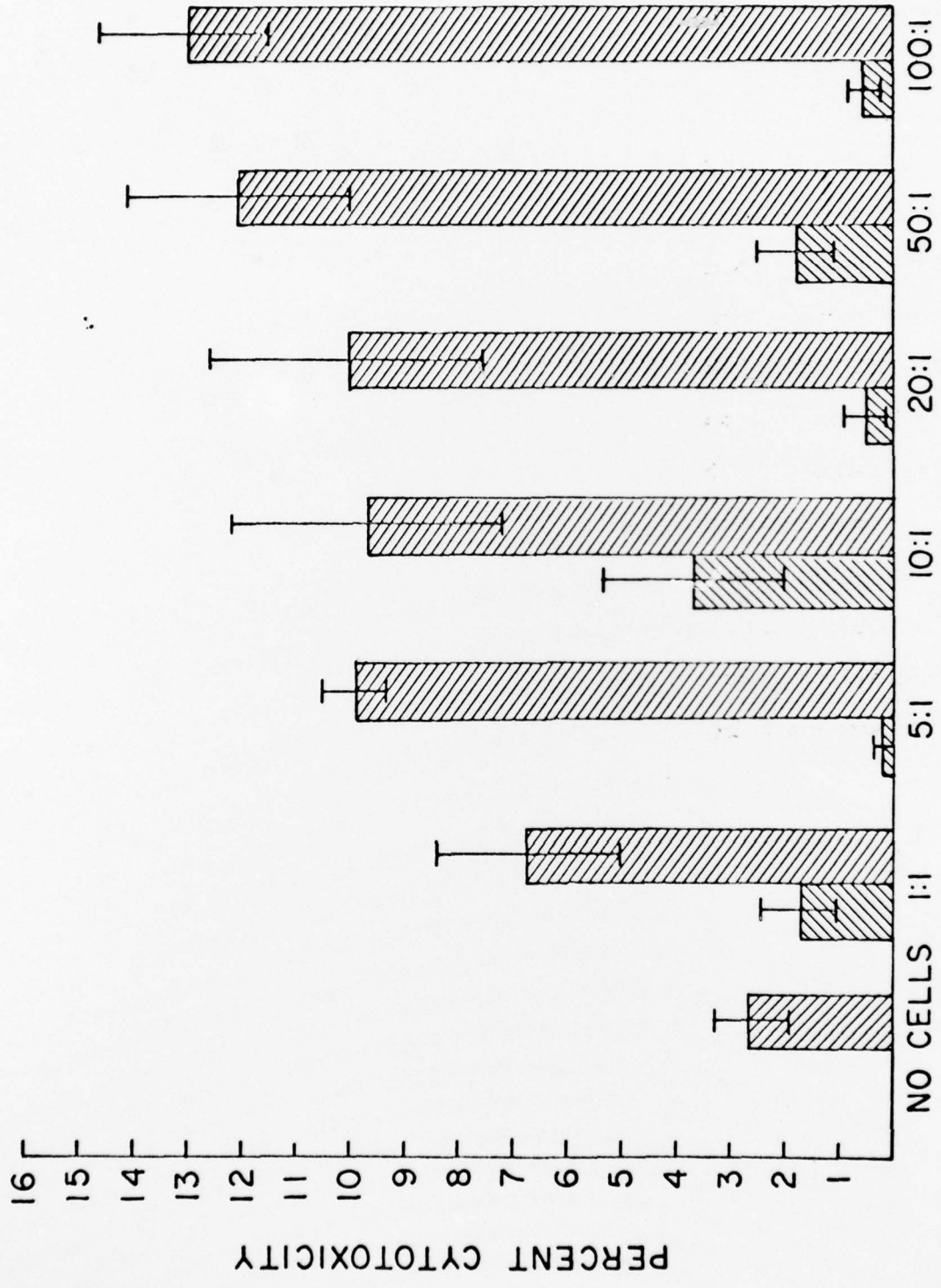
PERCENT CYTOTOXICITY
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
NO CELLS
1:1
5:1
10:1
20:1
50:1
100:1
MOUSE SPLEEN CELL TO TRYPANOSOME RATIO

Figure 1

Legend - Figure 2

The percent cytotoxicity of trypanosomes incubated in normal mouse serum with  or without  complement and in hyperimmune mouse serum with  or without  complement. Trypanosomes lysed by distilled water released 69.2% of the incorporated ^{3}H-leucine as compared with the total ^{3}H-leucine release found by drying trypanosomes on to filter paper disc. Distilled water lysed trypanosomes were not infective to mice and thus were equated to 100% cytotoxicity. Vertical bars represent $\pm$ the standard error ofthe mean for six experiments. The ratio of mouse spleen cells to trypanosomes was 20:1. Cytotoxicity observed with immune serum and normal spleen cells or immune serum and immune spleen cells was significantly different ($p < 0.01$) when compared to normal or immune serum without cells.

Legend - Figure 2

The percent cytotoxicity of trypanosomes incubated in normal mouse serum with  or without  complement and in hyperimmune mouse serum with  or without  complement. Trypanosomes lysed by distilled water released 69.2% of the incorporated ^{3}H-leucine as compared with the total ^{3}H-leucine release found by drying trypanosomes on to filter paper disc. Distilled water lysed trypanosomes were not infective to mice and thus were equated to 100% cytotoxicity. Vertical bars represent $\pm$ the standard error ofthe mean for six experiments. The ratio of mouse spleen cells to trypanosomes was 20:1. Cytotoxicity observed with immune serum and normal spleen cells or immune serum and immune spleen cells was significantly different ($p < 0.01$) when compared to normal or immune serum without cells.

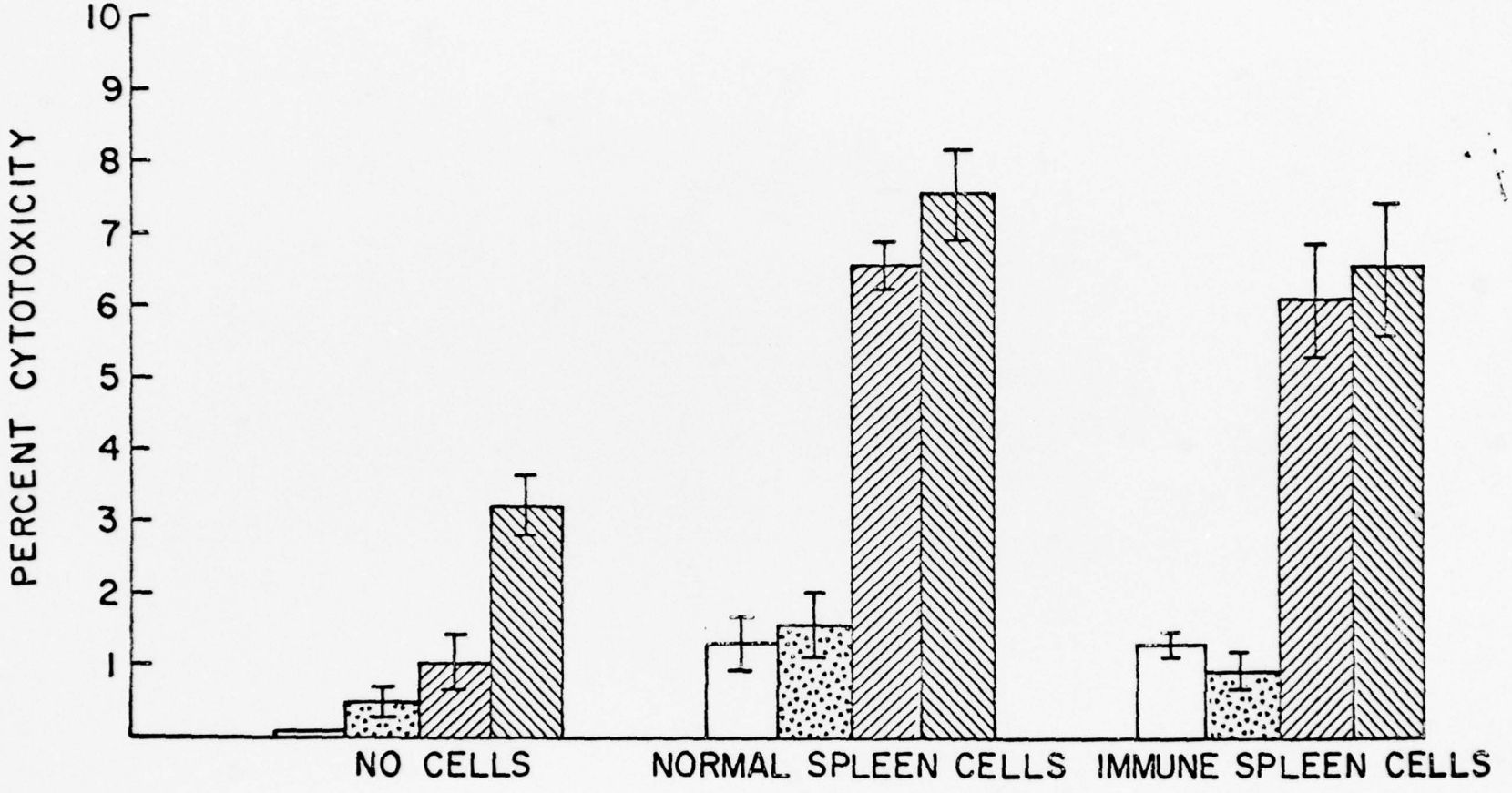
PERCENT CYTOTOXICITY
10
9
8
7
6
5
4
3
2
1
NO CELLS
NORMAL SPLEEN CELLS
IMMUNE SPLEEN CELLS

Figure 2

Legend - Figure 3

The effects of macrophage enriched lymphocyte enriched spleen cell populations in mediating cytotoxicity of T. rhodesience incubated in normal mouse serum  or in immune mouse serum  . Bars represent mean ± standard error of the mean for three experiments.

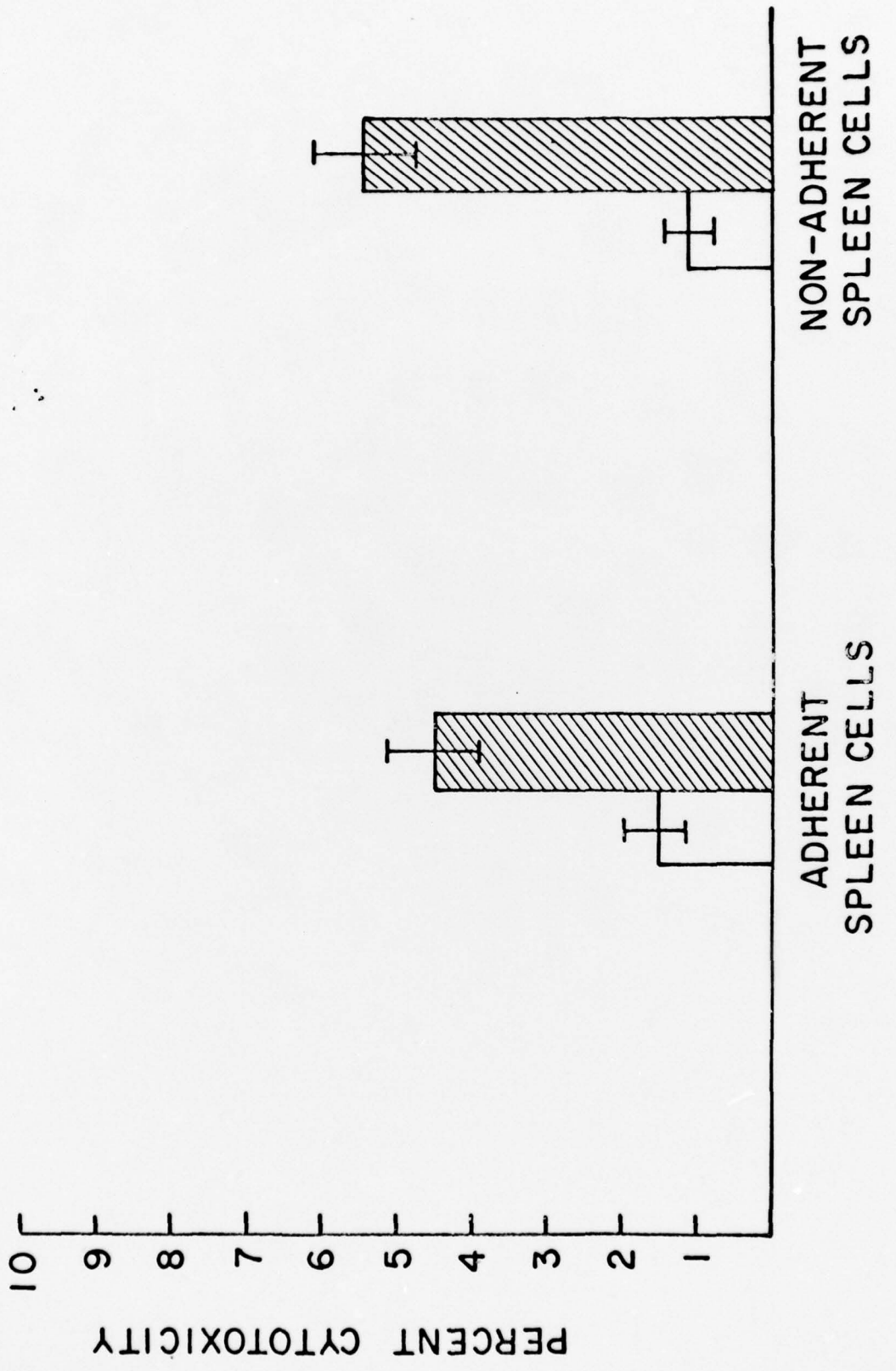
PERCENT CYTOTOXICITY
10
9
8
7
6
5
4
3
2
1
ADHERENT SPLEEN CELLS
NON-ADHERENT SPLEEN CELLS

Figure 3